

Superoxide dismutase, catalase, glutathione peroxidase and NADPH oxidase in lead-induced hypertension

NOSRATOLA D. VAZIRI, CHING-YI LIN, FARBOD FARMAND, and RAM K. SINDHU

Division of Nephrology and Hypertension Departments of Medicine, Physiology and Biophysics University of California, Irvine, Irvine, California, USA

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Background. Earlier studies from this laboratory have revealed the presence of oxidative stress and its role in the pathogenesis of lead-induced hypertension (HTN). We have further shown evidence of increased hydroxyl radical ($\cdot\text{OH}$) and superoxide production in lead-treated rats and cultured endothelial cells. This study was designed to determine whether oxidative stress in animals with lead-induced HTN is associated with dysregulation of the main antioxidant enzymes namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) or increased superoxide producing enzyme nicotinamide adenine dinucleotide (phosphate) oxidase [NAD(P)H].

Methods. Male Sprague-Dawley rats were randomly assigned to lead-exposed and control groups. Animals in the lead-exposed group were provided with drinking water containing 100 ppm lead acetate for 12 weeks. The control group was provided with regular drinking water. At the conclusion of the experiment, immunodetectable Cu Zn SOD, Mn SOD, CAT, GPX and gp⁹¹ phox subunit of NAD(P)H oxidase were determined by Western analysis in the kidney, brain and left ventricle of control and lead-exposed rats. Subgroups of the study animals were treated with IV infusion (180 $\mu\text{mol/kg/h}$) of the superoxide trapping agent, tempol, and arterial pressure and urinary nitric oxide (NO) metabolite (NO_x) excretion were determined.

Results. Lead exposure for 12 weeks resulted in a marked rise in systolic blood pressure, a significant reduction in urinary NO_x excretion, a significant increase in kidney and brain Cu, Zn SOD, a significant increase in brain and insignificant increase in kidney and heart gp⁹¹ phox. In contrast, Mn SOD, CAT and GPX in the kidney, brain and left ventricle were unchanged. Incubation with lead acetate did not alter SOD activity in vitro. Infusion of tempol significantly lowered arterial pressure and raised urinary NO_x excretion in the lead-exposed group (but had no effect in the control group) pointing to increased superoxide production in the lead-exposed animals.

Conclusion. Animals with lead-induced hypertension exhibited oxidative stress which was associated with mild up-regulation of superoxide-generating enzyme, NAD(P)H oxidase, with no evidence of quantitative SOD, CAT or GPX deficiencies.

Key words: lead, hypertension, oxidative stress, superoxide, superoxide dismutase, tempol, nitric oxide, catalase, glutathione peroxidase, NAD(P)H oxidase.

Received for publication May 9, 2002

and in revised form July 26, 2002

Accepted for publication August 12, 2002

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Chronic exposure to low levels of lead causes sustained arterial hypertension (HTN) that persists indefinitely after the cessation of lead exposure in humans and experimental animals [1–8]. In a series of earlier studies [1–5], we have demonstrated that lead-induced HTN is associated with oxidative stress and increased production of reactive oxygen species (ROS). We have further shown that the associated oxidative stress plays a major role in the pathogenesis of HTN via avid inactivation/oxidation of nitric oxide (NO) by ROS [1, 4]. In fact, alleviation of oxidative stress by a variety of antioxidants resulted in marked amelioration of HTN and enhanced NO availability in rats with lead-induced HTN [1, 3–5]. Avid inactivation of NO by ROS was invariably associated with a compensatory up-regulation of NO synthase (NOS) isoforms that was largely reversed by antioxidant therapy in lead-treated animals [3, 5].

In a series of earlier studies, we found strong evidence for increased hydroxyl radical ($\cdot\text{OH}$) in the lead-treated rats and cultured endothelial cells. For instance, using a salicylate trapping technique, we found a marked increase in $\cdot\text{OH}$ radicals in various tissues of lead-treated rats and in lead-treated cultured endothelial cells [9, 10]. The role of excess $\cdot\text{OH}$ activity was further confirmed by the observations that intravenous administration of the reputed $\cdot\text{OH}$ scavenger, dimethylthiourea (DMTU), resulted in a marked amelioration of HTN and increased urinary excretion of NO metabolites (NO_x) in rats with lead-induced HTN [10]. In contrast, intravenous administration of the native superoxide dismutase (SOD) alone or together with catalase had no discernible effect on either blood pressure or urinary NO_x excretion [11]. However, as a peptide, native SOD is cell impermeable and as such its lack of demonstrable action does not necessarily preclude increased cellular production of superoxide anion. In an attempt to overcome this obstacle, we recently used the cell-permeable SOD-mimetic agent, tempol, in a study designed to explore the effect of lead exposure on SOD production in cultured human coronary endothelial cells. The results revealed a marked

increase in lipid peroxidation product, malondialdehyde (MDA), denoting oxidative stress, coupled with a compensatory up-regulation of endothelial NO synthase (eNOS) expression in lead-treated endothelial cells. The study further revealed normalization of MDA production and eNOS expression by superoxide-trapping drug tempol in lead-exposed (but not control) cells [12]. The latter study provided strong evidence for increased superoxide production in lead-treated endothelial cells. These *in vitro* findings were subsequently confirmed in our *in vivo* studies, which showed amelioration of HTN and oxidative stress together with enhanced NO availability and reversal of compensatory up-regulation of NOS isoforms by tempol administration in rats with lead-induced HTN [5]. Since $\cdot\text{OH}$ is produced from interaction of superoxide and hydrogen peroxide (H_2O_2) via Fenton or Haber Weiss reactions, elevation of superoxide production or its reduced dismutation can readily account for increased $\cdot\text{OH}$ production in lead-treated cultured cells and in intact animals observed in our earlier studies [9, 10]. The present study was undertaken to explore whether oxidative stress in lead-induced HTN is due to down regulation of the main antioxidant enzymes, that is, superoxide dismutase (SOD) isoforms, catalase (CAT) and glutathione peroxidase (GPX), or up-regulation of the superoxide-generating enzyme, NAD(P)H oxidase.

METHODS

Animals

Male Sprague-Dawley rats with an average weight of about 200 g were housed in a climate controlled light-regulated space with 12-hour light (≈ 500 Lux) and dark cycles. They were fed a regular rat chow *ad libitum*. The animals were randomly assigned to lead-exposed and normal control groups. Animals in the lead-exposed group were provided with a drinking water containing 100 ppm lead acetate for 12 weeks. The control group was provided with regular drinking water.

At the conclusion of the observation period, tail arterial pressure was measured and animals were placed in metabolic cages for a timed urine collection. The next day, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and euthanized by exsanguination using cardiac puncture. Kidney, brain and heart were immediately harvested, cleaned, snap-frozen in liquid nitrogen and stored at -70°C until being processed. The procedures were carried out between the hours of 9 a.m. to 11 a.m.

An additional group of six lead-exposed and six control rats were instrumented and used for the assessment of changes in blood pressure and urinary NO_x excretion in response to a single IV infusion of tempol (Sigma Chemical Co, St. Louis, MO, USA) as described below.

Determination of response to intravenous tempol

Under general anesthesia with thiobutabarbital (100 mg/kg IP), the animals were instrumented as follows: A tracheostomy was performed and a PE-240 catheter was placed in the trachea. A PE-90 catheter with a flared tip was placed in the bladder for urine collection. In addition, PE-50 catheters were placed in the left carotid artery and right jugular vein for direct blood pressure measurement and intravenous infusions, respectively. After a 60-minute equilibration and a 30-minute saline infusion control period, animals were treated with continuous IV infusion of tempol (180 $\mu\text{mol/kg/h}$) for 30 minutes followed by a 30-minute recovery period during which saline was infused. All infusions were carried out at a rate of 2 mL/kg/h. Urine samples were collected during each period and blood samples were obtained at the end of the control, tempol treatment and recovery periods.

Arterial blood pressure was monitored directly via the carotid catheter that was connected to a Gould P-50 pressure transducer and recorded on a Dynograph R511A recorder (Sensor Medics, Anaheim, CA, USA) as previously described [13].

Measurements of SOD, catalase, glutathione peroxidase and gp⁹¹ phox proteins

Homogenates (25% wt/vol) of kidney, heart (left ventricle) and brain were prepared in 10 mmol/L HEPES buffer, pH 7.4, containing 320 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), 10 $\mu\text{g/mL}$ leupeptin, 2 $\mu\text{g/mL}$ aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) at 0 to 4°C with a polytron homogenizer. Homogenates were centrifuged at $9000 \times g$ for 10 minutes at 4°C to remove nuclear fragments and tissue debris without precipitating membrane fragments. A portion of the supernatant was used to determine the total protein concentration by using a Bio-Rad kit (Hercules, CA, USA).

Total cellular protein (1 μg for Cu, Zn SOD and 20 μg for Mn SOD, catalase, glutathione peroxidase and gp⁹¹ phox assays) was electrophoresed in 4 to 20% Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels (Novex, San Diego, CA, USA). Proteins were transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA, USA), blocked in 5% dry milk in T-TBS (0.02 mol/L Tris/0.15 mol/L NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature for three hours, washed three times with T-TBS and incubated with the primary antibodies to the Cu, Zn SOD, Mn SOD (1:1000), catalase (1:2000), glutathione peroxidase (1:250) and gp⁹¹ phox (1:2000) for three hours at room temperature. All of these antibodies were purchased from Calbiochem (San Diego, CA, USA). After washing five times with T-TBS, the blots were incubated with secondary antibodies (anti-sheep for Cu, Zn SOD, Mn SOD and glutathione peroxidase, 1:2000 and anti-rabbit for catalase 1:2000) conju-

Table 1. Systolic arterial blood pressure (BP), urinary NO_x excretion (UNO_x), serum creatinine and body weight in the normal control and lead-exposed groups

Groups	BP mm Hg	U NO _x μmol/5 h	Serum creatinine mg/dL	Creatinine clearance mL/min	Body weight g
Control	122 ± 8	1.38 ± 0.27	0.48 ± 0.02	2.1 ± 0.4	424 ± 27
Lead-Exposed	179 ± 11	0.76 ± 0.13	0.49 ± 0.04	2.3 ± 0.2	422 ± 26
<i>P</i> values	<0.01	<0.01	NS	NS	NS

N = 12 in each group; NS is not significant.

gated with horseradish peroxidase at room temperature for two hours. After washing five times with T-TBS, the membrane was developed using enhance chemiluminescent (ECL) reagent (Amersham Life Science Inc., Arlington Heights, IL, USA) and subjected to autoluminography for one to five minutes. The autoluminographs were scanned with a laser densitometer (Model PD 1211; Molecular Dynamics, Sunnyvale, CA, USA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain, to verify the uniformity of protein load and transfer efficiency across the test samples. Membranes failing the test were discarded and measurements were repeated.

Measurement of arterial pressure

Arterial pressure was determined by tail plethysmography (Harvard Apparatus, Natick, MA, USA) as described in our earlier studies [2].

Effect of lead on SOD activity

In an attempt to explore possible effects of lead on SOD enzymatic activity, purified SOD preparations from bovine erythrocytes (Sigma) were assayed in the presence of different concentrations (0, 0.1, 0.25, 2.5, and 25 μmol/L) of sodium acetate or lead acetate at room temperature.

In another series of experiments, purified SOD preparations, as well as rat liver cytosols were incubated in the presence of 0, 0.1, 0.25, 2.5 and 25 μmol/L sodium acetate or lead acetate for 20 hours at room temperature and SOD activity was then determined by using a kit purchased from R & D Systems (Portland, OR, USA).

Measurement of total nitrate and nitrite (NO_x)

The concentration of NO_x in the test samples was determined by means of the Sievers Instruments Model 270B Nitric Oxide Analyzer (NOA™; Sievers Instruments, Boulder, CO, USA) as described earlier [14].

Data analysis

Data are expressed as mean ± SEM. Analysis of variance (ANOVA), multiple range test and the Student *t* test were used as appropriate. *P* values less than 0.05 were considered significant.

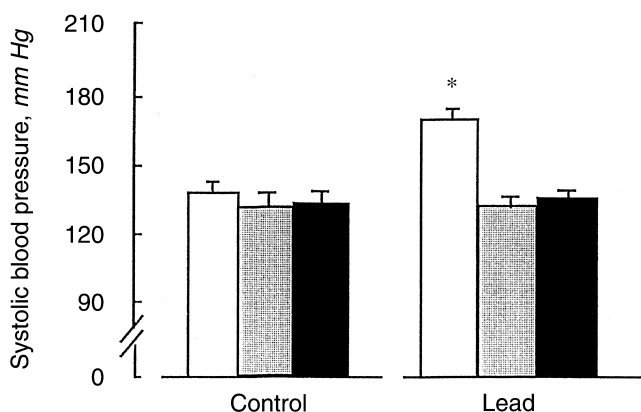


Fig. 1. Systolic blood pressure measurements obtained via a carotid catheter at baseline (□), following a 30-minute IV infusion of tempol (180 μmol/kg/h; ▨) and at the end of a 30-minute recovery period (■) in rats with lead-induced hypertension (lead) and control animals. *N* = 6 animals in each group. Data are given as $\bar{x} \pm \text{SEM}$. **P* < 0.01 (ANOVA).

RESULTS

General data

Compared to the control group, the lead-exposed rats exhibited a significant elevation of arterial pressure. Arterial pressure measurements obtained by tail plethysmography closely approximated values obtained by direct measurements via carotid artery catheters. Elevation of arterial blood pressure in the lead-exposed rats was coupled with a significant reduction in urinary NO_x excretion. No significant difference was found in either body weight, serum creatinine or creatinine clearance between the lead-exposed and the control groups at any point during the study period. Data are depicted in Table 1.

Effect of tempol infusion

Intravenous infusion of tempol in rats with lead-induced HTN resulted in a significant decline in arterial pressure to the values that were comparable to those seen in the normal control group. The effect of tempol on arterial pressure persisted during the 30-minute period following cessation of tempol infusion. In contrast, tempol infusion did not significantly alter arterial pressure in the control animals. The decline in arterial pressure during tempol infusion was coupled with a significant rise in urinary NO_x in rats with lead-induced HTN. As with arterial pressure, the effect of tempol on urinary NO_x excretion persisted during the 30-minute recovery period after cessation of tempol infusion in the lead-exposed animals. In contrast, tempol infusion had no effect on urinary NO_x excretion in the control group (data are shown in Figs. 1 and 2).

Tissue SOD isoforms

Data are depicted in Figures 3 to 5. The lead exposed animals exhibited a significant increase in immunodetect-

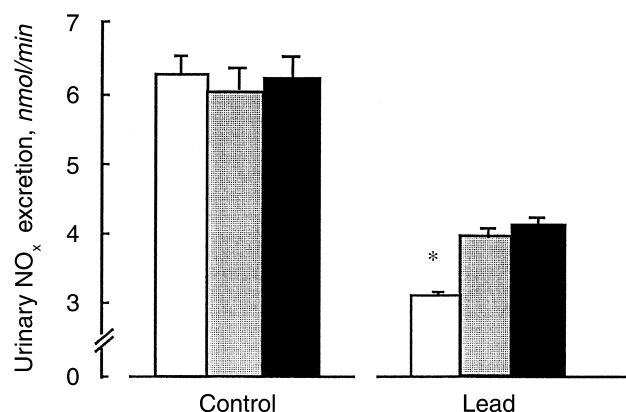


Fig. 2. Urinary excretion of nitric oxide metabolites (NO_x) obtained at baseline (□), during tempol infusion (▨), and recovery periods (30 minutes each; ■) in rats with lead-induced hypertension (lead) and control animals. *N* = 6 animals in each group. Data are shown as $\bar{x} \pm$ SEM. **P* < 0.01 (ANOVA).

able Cu, Zn SOD abundance in the kidney and brain tissues as compared with the corresponding values found in the control group. However, left ventricular Cu, Zn SOD abundance was similar in the two groups. Although the mean Mn SOD abundance in the left ventricle and brain tissues was higher in the lead-exposed animals, the difference did not reach statistical significance.

Effect of lead on SOD activity in vitro

No significant difference was found in the SOD activity when purified SOD preparations were assayed in the presence of 0, 0.1, 0.25, 2.5 and 25 μ mol/L sodium acetate (placebo) or lead acetate (Fig. 6A).

In another series of experiments, purified SOD preparations (Fig. 6B) as well as the rat liver cytosols (Fig. 6C) were incubated in the presence of 0, 0.1, 0.25, 2.5 and 25 μ mol/L sodium acetate (placebo) or lead acetate for 20 hours at room temperature. Once again, extended exposure to lead acetate had no effect on the SOD enzyme activity of the given preparations when compared to the sodium acetate or untreated group.

Tissue catalase and glutathione peroxidase data

Results are illustrated in Figures 3 to 5. No significant difference was found in either catalase or glutathione peroxidase abundance in any of the tissues tested.

Tissue gp⁹¹ phox data

Data are shown in Figure 7. gp⁹¹ phox protein abundance was significantly higher in the brain and insignificantly higher in the renal cortex and heart of the lead-treated group as compared with the control group.

DISCUSSION

Compelling evidence has recently emerged implicating the role of oxidative stress in the pathogenesis and main-

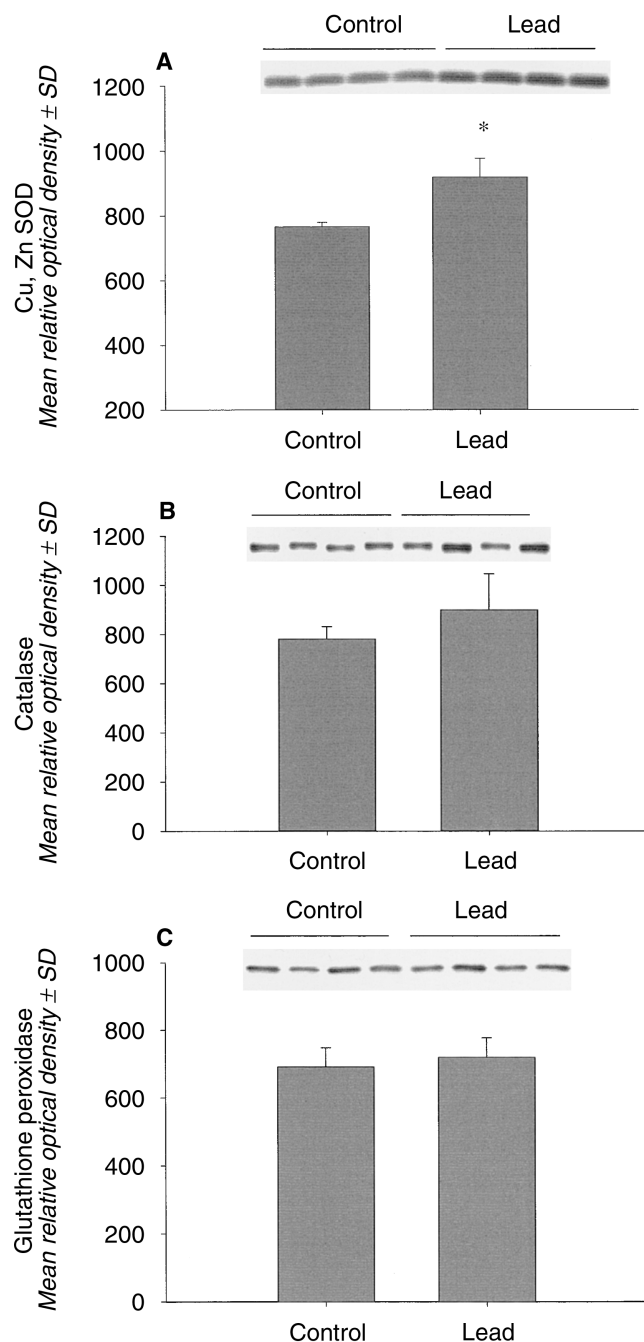


Fig. 3. Representative Western blot and group data for (A) Cu, Zn SOD, (B) catalase and (C) glutathione peroxidase protein abundance in the kidney of lead-treated and control rats. Values are presented as mean relative optical density \pm SD. **P* < 0.05.

tenance of various forms of genetic and acquired HTN. First, increased ROS activity has been found in animals and humans with various forms of HTN. These include rats with lead-induced HTN [1–4, 10, 11], chronic renal failure [15, 16], cyclosporine-induced HTN [17, 18], spontaneously hypertensive rats [19–23], salt-sensitive Dahl rats, [24, 25], rats fed a high-fat, high-sugar diet [26, 27],

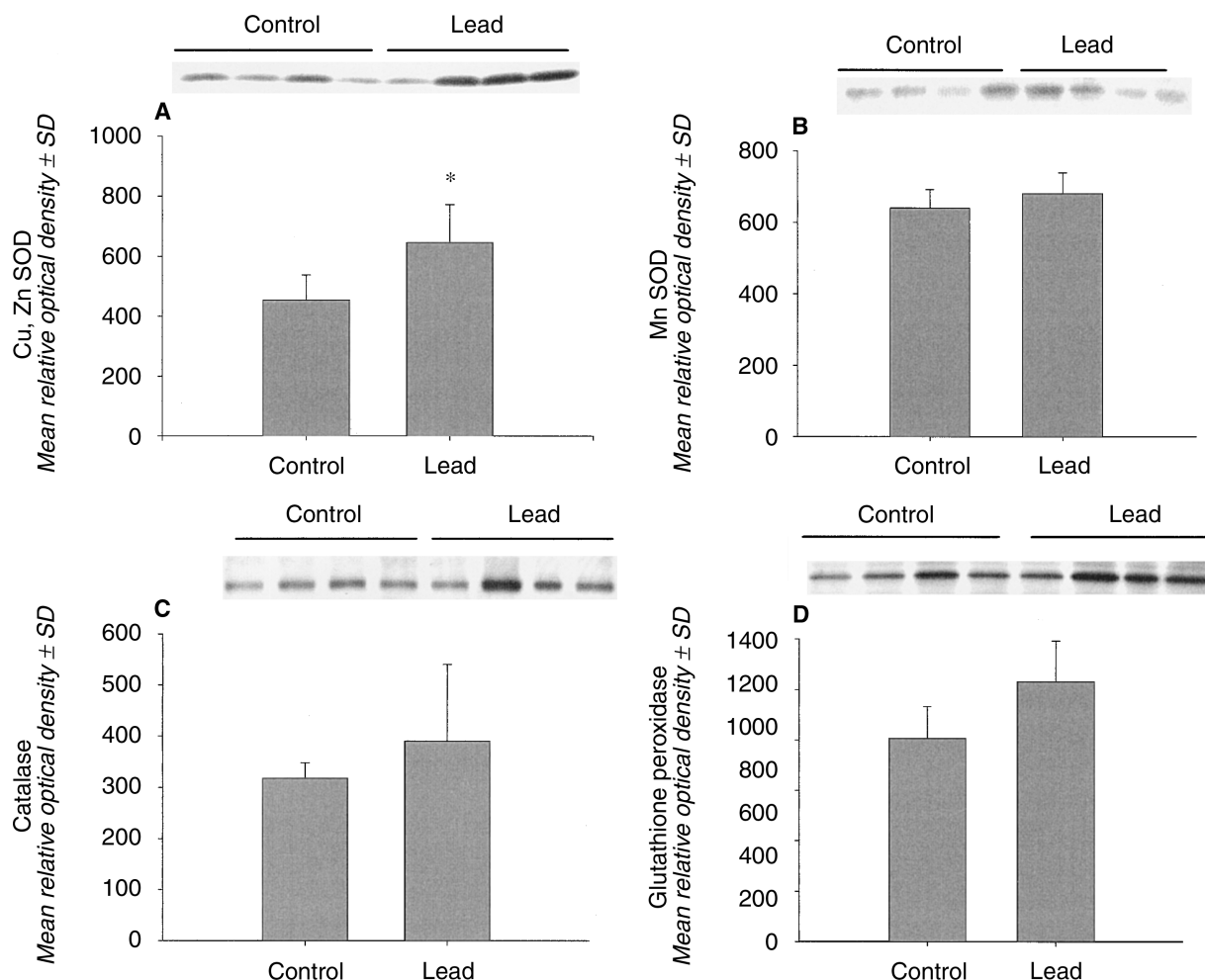


Fig. 4. Representative Western blot and group data for (A) Cu, Zn SOD, (B) Mn SOD, (C) catalase and (D) glutathione peroxidase protein abundance in the brain of lead-treated and control rats. Values are presented as mean relative optical density \pm SD. * $P < 0.05$.

and women with pre-eclampsia [28]. Second, alleviation of oxidative stress with a variety of antioxidant regimens has been shown to ameliorate HTN in several different forms of HTN [1–4, 15, 21–25], thus enforcing the notion that oxidative stress contributes to the maintenance of HTN. Finally, we recently showed that induction of oxidative stress by glutathione depletion causes severe sustained HTN in genetically normal, otherwise intact animals, thus, providing convincing evidence that oxidative stress, per se, can cause HTN [29].

In a series of earlier studies, we have shown that lead-induced HTN is associated with and is due, at least in part, to increased ROS activity [1–4, 10, 11]. The studies further showed evidence for increased superoxide [5, 12] and hydroxyl radical production in lead-exposed rats and cultured endothelial cells [10, 11].

Intravenous infusion of tempol, which has been shown to trap superoxide [30, 31], resulted in normalization of arterial pressure in rats with lead-induced HTN employed in the present study. This was accompanied by

a rise in urinary NO_x excretion pointing to improved NO availability, which could account, at least in part, for the fall in blood pressure in lead-exposed animals. In contrast to the lead-exposed animals that showed a dramatic fall in arterial pressure and a rise in urinary NO_x excretion, normal control animals exhibited no discernible response to tempol administration. This observation argues against a nonspecific effect of tempol on either arterial pressure or urinary NO_x excretion and points to increased abundance of superoxide in rats with lead-induced HTN.

While the presence of oxidative stress in lead-induced HTN is well established, its underlying mechanism is unknown. Oxidative stress can occur as a result of either increased ROS generation, depressed antioxidant system or both. The natural antioxidant system consists of a series of antioxidant enzymes and numerous endogenous and dietary antioxidant compounds that react with and inactivate ROS. The primary ROS produced in the aerobic organisms is superoxide that is a highly reactive and

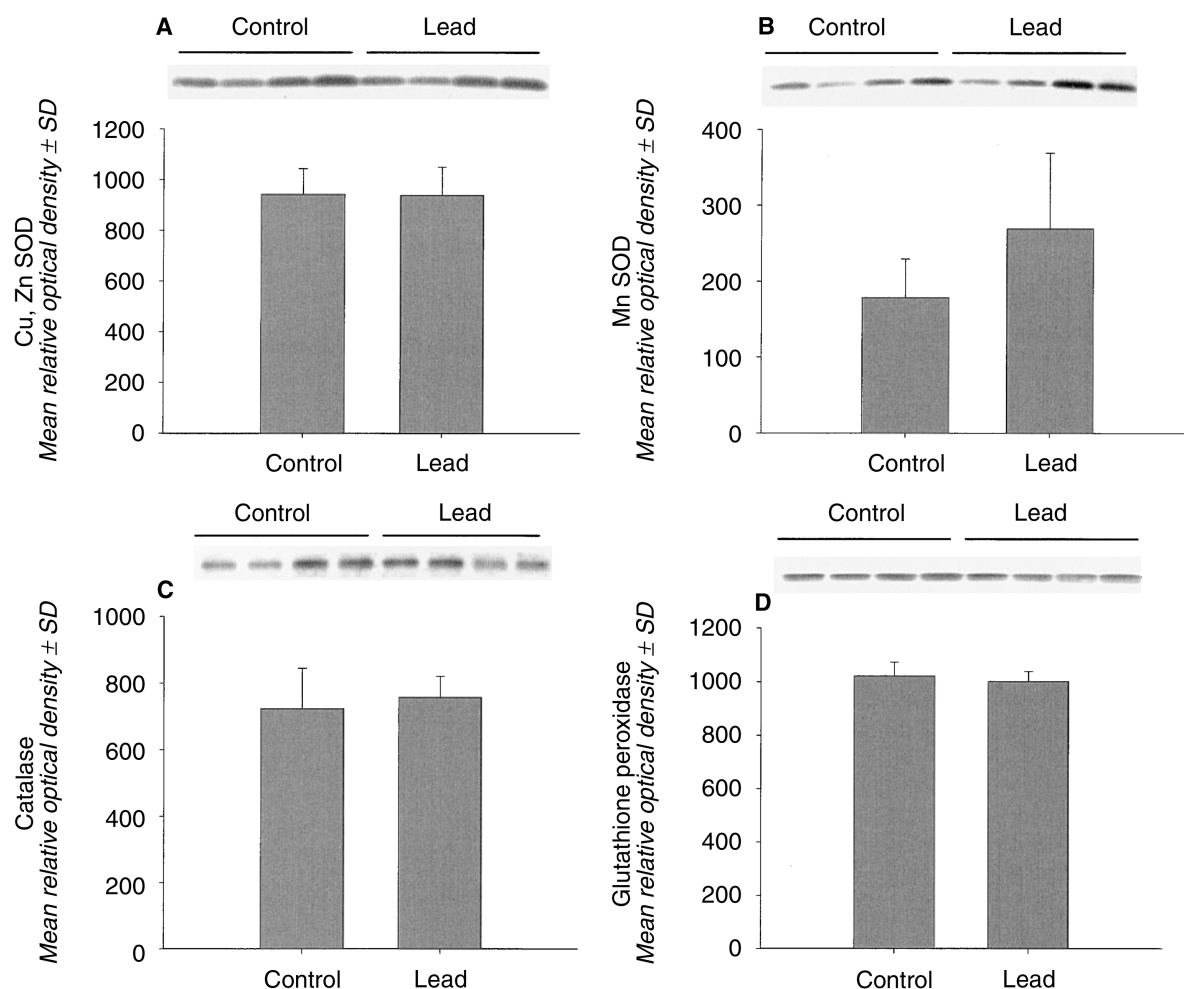


Fig. 5. Representative Western blot and group data for (A) Cu, Zn SOD, (B) Mn SOD, (C) catalase and (D) glutathione peroxidase protein abundance in the heart of lead-treated and control (CTL) rats. Values are presented as mean relative optical density \pm SD.

cytotoxic agent. Superoxide is converted to H_2O_2 by a group of enzymes known as SOD. H_2O_2 , in turn, is converted to water and molecular oxygen by either CAT or GPX. In addition, GPX can reduce lipid peroxides and other organic hydroperoxides that are highly cytotoxic products. Accordingly, SOD, CAT and GPX constitute the principal components of the antioxidant defense system and their deficiencies can cause oxidative stress. The present study, therefore, was conducted to determine the effect of chronic exposure to low levels of lead that causes longstanding HTN and oxidative stress on the abundance of these enzymes. The study revealed a mild but significant increase in immunodetectable Cu, Zn SOD abundance in the kidney and brain of rats with lead-induced HTN. However, immunodetectable Mn SOD abundance was not significantly altered in the lead-exposed animals. These findings clearly exclude a quantitative deficiency of intracellular SOD isoforms in this model. Moreover, in vitro experiments revealed no sig-

nificant change in the enzymatic activity of SOD after incubation with different concentrations of lead acetate.

Catalase is a tetrameric peroxidase enzyme which converts H_2O_2 to water and molecular oxygen and whose gene expression is regulated by H_2O_2 [32]. CAT plays an important role in ROS metabolism and in adaptation to oxidant stress [32]. Immunodetectable CAT abundance in the kidney, brain and left ventricle of rats with lead-induced HTN was similar to that found in the control animals. Thus, chronic exposure to low levels of lead does not appear to affect tissue CAT abundance in this model.

Glutathione peroxidase is a selenium-containing tetrameric enzyme that reduces H_2O_2 , lipoperoxides and organic hydroperoxides using glutathione as a hydrogen donor. Gene expression of GPX is up-regulated by H_2O_2 and other ROS [32]. As with CAT, tissue abundance of GPX was not altered in rats with lead-induced HTN.

Based on our findings, oxidative stress in lead-induced HTN does not appear to be due to a primary down regula-

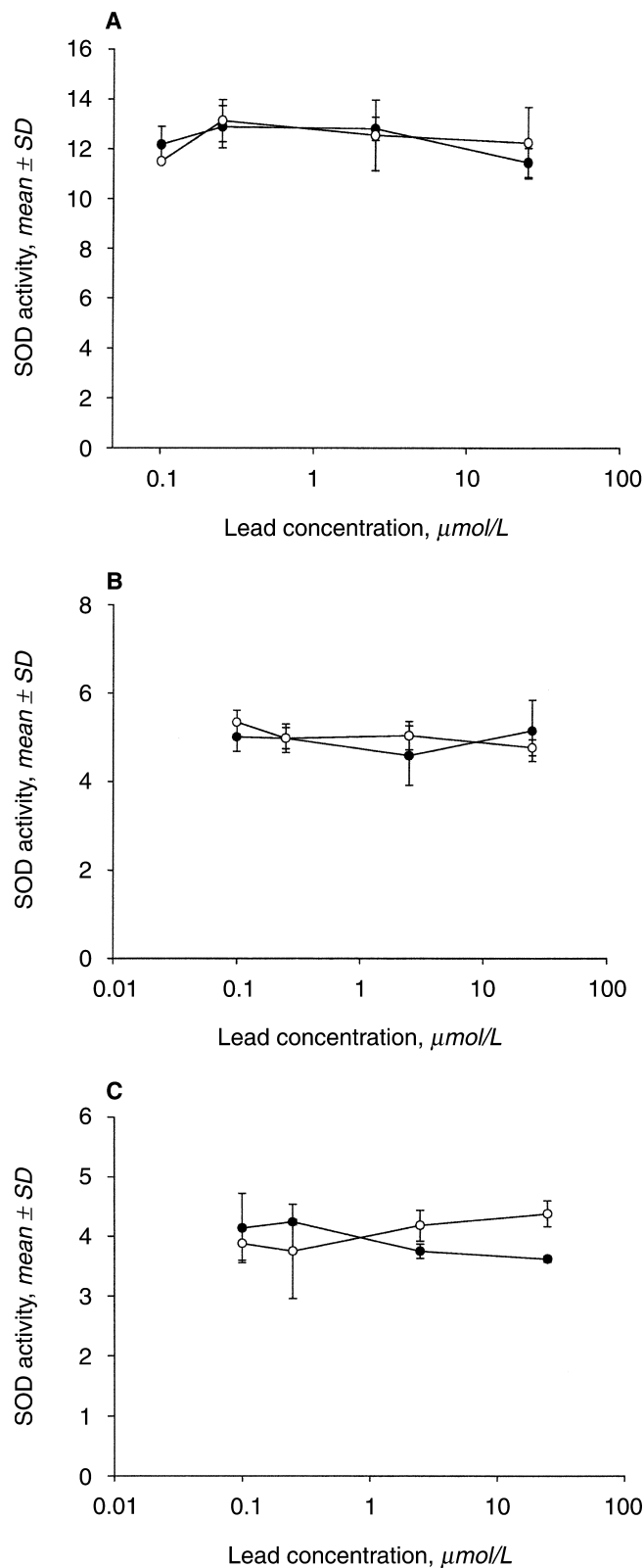


Fig. 6. Purified Cu, Zn SOD preparations from bovine erythrocytes (Sigma) were assayed in the presence of various concentrations of sodium acetate (placebo; ○) or lead acetate (●; A). In another series of experiments, purified Cu, Zn SOD preparations (B) or control rat liver cytosols (C) were incubated in the presence of various concentrations of sodium

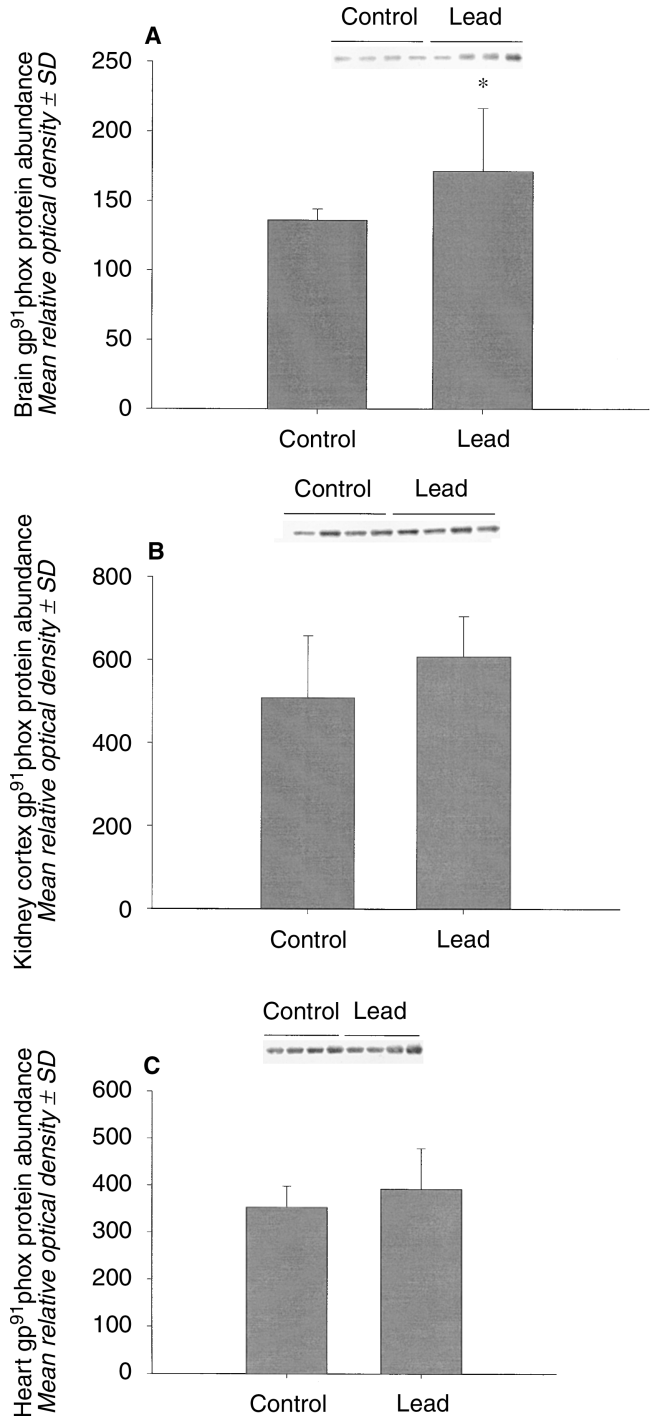


Fig. 7. Representative Western blots and group data depicting gp⁹¹phox protein abundance in the (A) brain, (B) renal cortex and (C) heart of lead-exposed and control rats. *N* = 6 in each group, **P* < 0.05.

acetate (placebo) or lead acetate for 20 hours at room temperature and the enzyme activity was then measured by using a kit purchased from R&D Systems. One SOD unit is defined as the activity that doubles the auto-oxidation rate of the control blank (*V*_s/*V*_c = 2) as described in the manufacturer's directions. The x-axis is presented on a logarithmic scale.

tion of either SOD isoforms, CAT or GPX. In fact, Cu, Zn SOD was mildly elevated and Mn SOD, CAT and GPX were normal in this model. The reason for the observed increase in the Cu, Zn SOD abundance in rats with lead-induced HTN is not clear. However, it may represent a compensatory response to oxidative stress that is known to augment SOD expression [32]. Since the antioxidant enzymes are regulated by ROS, the lack of up-regulation of CAT and GPX despite the presence of oxidative stress may represent an inappropriate response. In view of the absence of a detectable primary deficiency of the main antioxidant enzymes in the lead treated animals, we sought to explore expression of the main ROS generating enzyme, namely NAD(P)H oxidase, in this model. This enzyme has been recently recognized as an important source of superoxide in cardiovascular tissues [33, 34]. Moreover, increased NAD(P)H oxidase-derived superoxide has been implicated in the pathogenesis of angiotensin II-induced hypertension [33]. Our rats with lead-induced hypertension exhibited a significant increase in the immunodetectable gp⁹¹ phox subunit of NAD(P)H oxidase in the brain and insignificantly higher levels in the heart and renal cortex. These observations suggest that increased superoxide activity in the lead exposed animals, in part, may be mediated by up-regulation of NAD(P)H oxidase. More comprehensive studies are planned to explore this possibility.

In conclusion, rats with lead-induced HTN exhibit oxidative stress that is associated with mild up-regulation of superoxide generating enzymes, NAD(P)H oxidase with no evidence of quantitative SOD, CAT or GPX deficiencies.

ACKNOWLEDGMENT

The authors are grateful to Ms. Carmen Eaton for her technical assistance with this paper.

Reprint requests to N.D. Vaziri, M.D., MACP, Division of Nephrology and Hypertension, Department of Medicine, UCI Medical Center, 101 The City Drive, Bldg. 53, Room 125, Rt. 81, Orange, California 92868, USA.

E-mail: ndvaziri@uci.edu

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